J Appl Biomed
DOI: 10.32725/jab.2022.006
journal homepage: http://jab.zsf.jcu.cz

Journal of Applied Biomedicine

Original research article

Exploring the therapeutic potential of acetonic plant extracts in the healing of skin wounds infected with multidrug resistant pathogens

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Abstract
Open wounds are easily susceptible to infection by multi-drug resistant (MDR) pathogens. The emergence of MDR super bacteria such as *Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus* spp, fungi such as *Aspergillus niger* and *Candida* spp, has been identified to significantly increase the incidence rate. Therefore, it is necessary to develop a suitable barrier to prevent infection and enhance wound healing. On the other hand, medicinal plants could represent a significant source of new antimicrobial drugs for combating MDR pathogens. Out of 60 clinical skin burn cases, 51 patients (85%) had polymicrobial infections, while the remaining had monomicrobial infections. *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*, and *Klebsiella pneumonia* were identified as the most common bacterial isolates based on morphological and biochemical tests. However, *Candida albicans, Candida parasitosis, Candida glabrata, Candida famata, Aspergillus niger*, and *Exophilia spinifera* were the most common fungal isolates found in skin burn cases. MDR classification was reported in 21 of the 39 bacterial isolates and 8 of the 27 fungal isolates. The antimicrobial activity of tested acetonic plant extracts rosemary, henna, and licorice against MDR isolates was compared to the commercial antibiotic agents. Acetonic rosemary extract outperformed henna and licorice extracts in antibacterial activity, while licorice extract outperformed henna and rosemary extracts on antifungal activity. As a result, rosemary and licorice extracts were chosen to prepare a topical cream for further *in vivo* wound healing and histopathology. Based on the antimicrobial potential of acetonic plant extracts against MDR isolates, BI-41 and FI-17 were chosen for *in vivo* wound healing. BI-41 stands for the molecularly identified species *Pseudomonas aeruginosa* SSM-15, while FI-17 stands for molecularly identified species *Aspergillus niger* SSM-27. *In vivo* testing showed that both cream formulas had excellent healing properties when administered topically. *In vivo* histopathological examination revealed that acetonic rosemary and licorice extract could be promising for wound healing, combating MDR pathogens of burn wound infections.

Keywords: Antimicrobial activity; Herbal plant extracts; Multidrug resistance; Topical cream; Wound healing

Highlights:
- Drug resistance has limited the use of conventional antibiotics.
- Acetonic rosemary and licorice extracts demonstrated effective antimicrobial activities.
- Plant extracts used in wound healing are safe and effective.

Introduction

The human skin acts as an anatomical barrier, shielding internal organs from external damage (Pereira et al., 2022). Wound healing is when damaged tissue in living organisms is replaced (Moholkar et al., 2021). Wound dressing materials such as hydrogels are commercially available (Peng et al., 2022). Despite this, wound care management remains difficult due to the risk of infection with multi-drug resistant (MDR) pathogens (Ali et al., 2016, 2017, 2018, 2020a, b, 2021; El Shafay et al., 2016; Khalil et al., 2022c). Plant extract-mediated wound dressing is gaining popularity due to its lower toxicity and fewer side effects, inherent medicinal properties, environmental sustainability, ease of availability at a lower cost, and good alternatives for wound management (Ali et al., 2020a, El-Zawawy and Ali, 2016; Nieto et al., 2018; Veenstra and Johnson, 2021). Skin infection is one of the most common causes of wound complications, and it occurs due to a favorable environment for pathogen growth. The emergence of MDR infections increases the difficulty of developing advanced wound dressings with potent antimicrobial activity and significant wound healing proper-

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http://doi.org/10.32725/jab.2022.006
Submitted: 2021-12-15 • Accepted: 2022-05-26 • Prepublished online: 2022-06-06
J Appl Biomed 20/2: 45–55 • EISSN 1214-0287 • ISSN 1214-021X

ties. Several studies have looked into the antimicrobial activity of natural compounds and medicinal plant extracts against common bacterial and fungal pathogens found in wounds, such as *Pseudomonas aeruginosa, Staphylococcus aureus, Candida* spp, and *Aspergillus niger* (Ali et al., 2017; El-Shouny et al., 2018; El Zawawy et al., 2020; Khalil et al., 2015; Sonbol et al., 2015).

Antibacterial and antifungal bioactive compounds found in medicinal plants can help wounds heal faster and more effectively (Ali et al., 2020a, 2022; Khalil et al., 2020). These compounds are made from plant parts such as leaves, stems, roots, bark, and fruits (Al-Tohamy et al., 2018). There has been a lot of interest in the mechanism of herbal medicines and purely natural products in wound healing in recent years. Some herbal medicines appear to act through multiple mechanisms and exhibit healing properties at various stages of the wound healing process (Gorain et al., 2022). According to different *in vitro* and *in vivo* experiments, many herbal extracts have significant antioxidant properties. Herbal plants' metabolic activities have the potential to yield high-value medicinal formulations. Tannins, alkaloids, carbohydrates, terpenoids, steroids, and flavonoids of physiologically active components (Ali et al., 2020a). Flavonoids and anthraquinones are powerful antioxidants. Ellagic acid, shikonin, and some herbal extracts have high antioxidant activity by scavenging reactive oxygen species (ROS), inhibiting lipid peroxidation, and increasing antioxidant enzyme intracellular activity. Herbal medicine also promotes fibroblast cell proliferation and has immunomodulatory and anti-inflammatory properties, which help speed up wound healing (Wadhwa et al., 2022).

For example, licorice (*Glycyrrhiza glabra* L.) contains over 20 triterpenoids and nearly 300 flavonoids. The main active components with antimicrobial properties are glycyrrhizin, licochalcone A, licochalcone E, and glabridin (Adamczak et al., 2019). Rosemary (*Rosmarinus officinalis* L.), on the other hand, contains a variety of compounds, including flavonoids, diterpenes, and polyphenols, all of which have biological bioactivities such as antibacterial, antifungal, antioxidant, and insecticide properties (Liu et al., 2017; Nieto et al., 2018). As a result, this study aimed to evaluate the *in vitro* antimicrobial activity of acetonic extracts of selected Egyptian medicinal plants such as *Glycyrrhiza glabra* and *Rosmarinus officinalis* against opportunistic MDR bacterial and fungal pathogens such as *Pseudomonas aeruginosa* and *Aspergillus niger*. Furthermore, *in vitro* cytotoxicity against fibroblast cells was assessed. Based on *in vivo* histopathological examination, it also investigated the efficacy of licorice and rosemary acetonic extracts on wound healing of experimental animals with skin burns. The findings of this study open up a new avenue for screening and characterizing new bioactive metabolites derived from medicinal plants as alternative antimicrobial agents with therapeutic potential.

Materials and methods

Plant leaves and acetone extraction preparation

The leaves of three plants used in this study, rosemary (*Rosmarinus officinalis* L.), henna (*Lawsonia inermis* L.), and licorice (*Glycyrrhiza glabra* L.), were dried at room temperature and ground into powder. To prepare the acetone extracts of these plants, 20 g of each dried powdered sample was steeped for 48 h at room temperature in 500 ml of acetone (100%) according to the experimental design (Suppl. Fig. 1). After that, the samples were sieved through Whatman No. 1 filter paper. To prevent degradation, the filtrates were vaporized to dryness at room temperature using a rotary evaporator and stored at 18 °C. To determine antibacterial activity, a stock solution of 100 mg plant extract in 1 ml dimethyl sulfoxide (DMSO) was prepared. The extracts were sterilized by filtration through 0.45 µm Millipore filters and stored at 4 °C until used.

Clinical specimen collection, microbial isolation, and identification

One hundred and twenty swab samples were collected from 60 clinical skin burn cases admitted to the outpatient Dermatology and Venereology clinic at Tanta University Hospitals in Egypt. The collected samples were transported aseptically to the Microbiology Laboratory, Faculty of Science, Tanta University, and inoculated on selective and non-selective culture media for further isolation and identification experiments. The isolation and identification of microorganisms were carried out as shown in Suppl. Fig. 1. For bacterial isolation, each sample was cultured on blood agar, cetrimide agar, MacConkey agar, and nutrient agar (Ali et al., 2019a; Amer et al., 2022; Morsy et al., 2017), while fungal isolation was done on Sabouraud's Dextrose agar (SDA) and Potato Dextrose agar (PDA) media, supplemented with 0.4 mg/ml chloramphenicol and 0.5 mg/ml (Ali and Sun, 2015; Ali et al., 2017). All plates were incubated for up to 72 h under aerobic conditions. Moderate or heavy growth was a positive culture, whereas sparse growth was negative. Individual strain morphology were identified and purified using several subcultures on fresh agar plates, and all stock cultures were kept at –70 °C in 50% glycerol. For bacteria and fungi, phenotypic identification and conventional biochemical tests were carried out (Ali and Sun, 2015, 2019; Ali et al., 2019b, c). Furthermore, isolated yeasts were identified using the protocols (Al-Tohamy et al., 2021). The isolates were biochemically identified using VITEK®2 automated systems (BioMérieux, Marcy-L'Étoile, France). The partial or nearly full-length 16S rRNA gene was used for bacteria, while fungi used 18S ribosomal small subunit RNA and the D1/D2 domain of the 26S ribosomal large subunit RNA. According to the manufacturer's instructions, the TaKara MiniBEST Extraction Kit Ver.3.0 and Dr. GenTLER (TakaRa, Japan) were used to extract genomic DNA. Primers and polymerase chain reaction (PCR) amplification conditions have previously been reported (Ali et al., 2019d; De Filippis et al., 2017). MEGA 7 was used to create a phylogenetic tree.

Antibiotic susceptibility and MDR selection

A modified Kirby–Bauer single-disk diffusion technique was used to test antibiotic susceptibility. For bacteria and fungi, respectively, Müller–Hinton and SDA agar plates were prepared. For inoculum preparation, the microbial solution was adjusted to a concentration of 1×10^7 CFU/ml. In this experiment, 17 commercially available antibacterial discs (amoxicillin, oxacillin, ampicillin, imipenem, cephalothin, cefuroxime, cefotaxime, trimethoprim, levofloxacin, chloramphenicol, erythromycin, rifampin, tetracycline, doxycycline, vancomycin, gentamicin, and nitrofurantoin) and 7 antifungal agents (fluconazole, ketoconazole, amphotericin B, metronidazole, itraconazole, and nystatin – all from Sigma Chemical Co., USA) were tested against the selected bacterial and fungal isolated from skin burn infection. The antibiotic susceptibility test was conducted on Müller–Hinton agar (MHA), and the diameter of the inhibition zone (IZD) surrounding the discs was assessed to evaluate sensitivity. The Clinical and Laboratory Standards Institute recommendations were used to interpret the data (CLSI, 2014). Resistance to at least three antimicrobial categories was defined as MDR.

Antimicrobial activity of different acetonic leaves extracts

In vitro antibacterial and antifungal activity of rosemary, henna, and licorice acetonic extracts were determined (Suppl. Fig. 1). The agar well diffusion technique was used to evaluate antibacterial activity. Bacterial and fungal suspensions of 1×10^7 CFU/ml were inoculated with sterilized MHA media. To produce the spore suspension, 0.5 ml of about 1×10^7 CFU/ml of bacteria was combined with 9.5 ml of sterile SDA medium. Each well received 50 µl of each acetonic plant extract (400 µg/ml). The positive control was gentamicin (15 µg/ml), and the negative control was acetone solvent. After incubation, the IZDs were determined, and the findings were interpreted by the Clinical and Laboratory Standards Institute (CLSI, 2014). Each test was performed in triplicate. The minimum inhibitory concentration (MIC) was determined using the serial dilution approach described by Ali et al. (2017). Various concentrations of acetonic plant extract (100–400 µg/ml) were prepared, and 2 µl of the prepared inoculum solution was added to each well. The plates were incubated at bacteria and fungi- optimal temperatures. The minimum inhibitory concentration (MIC) was the lowest concentration at which no visible growth occurred. SEM was used to investigate the mechanism of antimicrobial action of acetonic plant extracts (Khalil et al., 2021). However, only the SEM results of *Glycyrrhiza glabra* acetonic extract antimicrobial action against *A. niger* SSM-27 are reported in this study.

Gas chromatography Mass Spectroscopy (GC-MS) analysis

Acetone extracts of rosemary and licorice were concentrated in a desiccator and subjected to GC-MS analysis (Khalil et al., 2022a, b). Gas chromatography coupled with mass spectroscopy (GC-MS, Shimadzu) was used to identify metabolic products. Temperatures were set at 270 °C for the injection port and 280 °C for the GC-MS interface, with a temperature flow rate of 10 °C/min. With 10 ml/min flow rate, helium was used as collision gas. The test samples' compound names and molecular formulas were determined using NIST library spectra databases.

In vivo *efficacy of a topical formulated cream as a wound-healing agent*

Cream preparation

Two topical creams were formulated using the acetonic rosemary and licorice extracts according to Purushothamrao et al. (2010) in the Laboratory of Pharmaceutical Technology, Faculty of Pharmacy, Tanta University, Tanta, Egypt, as previously described by El-Shouny et al. (2014). The cream formulation was evaluated on the skin of diseased and injured mice. At a concentration of 1×10^7 CFU/ml, *P. aeruginosa* SSM-15 and *A. niger* SSM-27, two MDR bacterial and fungus strains were employed to create the inoculum for infection in experimental animal models.

Animals

The animal experiment protocol was carried out by the Institutional Animal Ethics Committee guidelines (IAEC), Faculty of Science, Tanta University, Egypt. In wound healing experiments, twenty-one mice (25–30 g each) were used to evaluate the acetonic rosemary and licorice extracts.

Healing wound model

The mice were divided into seven groups (n = 3) at random. Group G1 (negative control) mice were burned but did not become infected or receive any treatment. Mice in Groups G2 and G3 (positive control, placebo cream receiving group) were burnt and infected with *P. aeruginosa* SSM-15 (G2) or *A. niger* SSM-27 (G3) at a sub-lethal dose of 1×10^7 CFU/ml/mouse. After three days of infection, the infected regions were covered in plastic film and treated twice daily for 17 days with placebo cream. Groups G4 and G5 (fusidic acid or nystatin-treated mice) were burnt, infected with *P. aeruginosa* SSM-15 or *A. niger* SSM-27 at a sub-lethal dosage of 1×10^7 CFU/ml/mouse, and treated with fusidic acid for bacterial infection (G4) or nystatin for fungal infection (G5). Burned mice were infected with *P. aeruginosa* SSM-15 or *A. niger* SSM-27 at a sub-lethal level of 1×10^7 CFU/ml/mouse and treated with acetonic rosemary extracts for bacterially infected mice (G6), or acetonic licorice extracts for fugally infected mice (G7).Untreated wounds were exposed to the open air, and the animals were housed in separate cages. The progress of healing lesions, restoring normal skin structure with topical wound therapy, and growing hairs on repaired skin were documented.

Histopathological examination

On day 17, skin biopsies were taken for histopathological studies. The biopsy samples (0.5×1.5 cm^2) were fixed in a buffered formaldehyde solution (10%). Paraffin block sections were prepared and stained with hematoxylin and eosin (H&E) for histopathological examinations. The microscopic findings were recorded regarding re-epithelialization, maturation and organization of epidermal squamous cells, granular cell layer thickness, and tissue development.

Scanning electron microscope

The effect of licorice acetonic extracts on *A. niger* (conidia, cell wall, and mycelia) was investigated using a scanning electron microscope (SEM) at Tanta University's Faculty of Medicine. The selected isolates were cultured PDA medium supplemented with tested plant extract at 400 µg/ml, or media without any supplements. A scanning electron microscope (Model JEOL, JSM-5200LV) was used to investigate it.

Statistical analysis

All experiments were carried out in triplicate, and the results were analyzed using NCSS 2020 (LIC, Utah, USA). A one-way analysis of variance (ANOVA) with an unpaired t-test was used to determine statistical significance at a P-value of 0.05. The P-values for notable comparisons were labelled with as follows, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

Results

Incidence and MDR pathogens recovered from skin burn wounds

Out of 60 clinically skin burn cases, 51 patients (85%) had polymicrobial infections, while the remaining cases had monomicrobial infections. Mixed bacteria alone were isolated from 26 (50.9%) of the 51 patients, combined fungi independently were separated from 15 (29.4%), and mixed bacteria and fungi were isolated from 10 patients (19.6%). *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*, and *Klebsiella pneumoniae* were identified as the most common bacterial isolates, based on morphological and biochemical tests. However, *Candida albicans, Candida parasitosis, Candida glabrata, Candida famata, Aspergillus niger*, and *Exophilia spinifera* were the most common fungal isolates found in skin burn cases.

All bacterial isolates were tested against 17 commercially available antibacterial discs (amoxicillin, oxacillin, ampicillin, imipenem, cephalothin, cefuroxime, cefotaxime, trimethoprim, levofloxacin, chloramphenicol, erythromycin, rifampin, tetracycline, doxycycline, vancomycin, gentamicin, and nitrofurantoin). 21 of the 39 bacterial isolates were classified as MDR. The prevalence of antibiotic resistance among the 21 MDR bacterial isolates is depicted in Fig. 1. All isolates were resistant to amoxicillin, ampicillin, cephalothin, cefuroxime, trimethoprim, and doxycycline. However, no MDR isolates were identified to be resistant to oxacillin or vancomycin (Fig. 1).

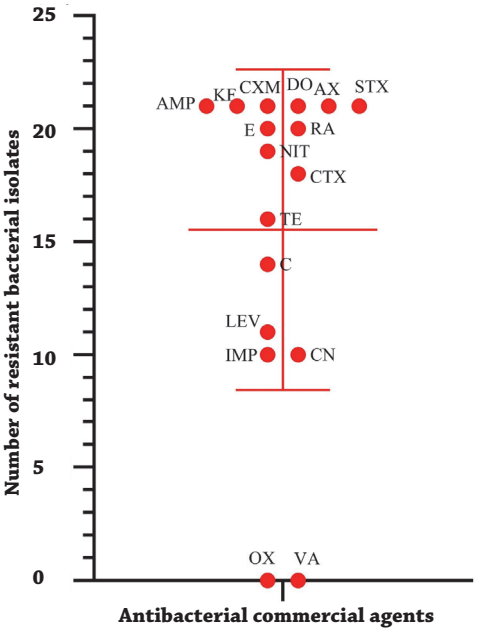

Fig. 1. The susceptibility of the 21 bacterial MDR pathogens to 17 commercially available antibacterial discs. AX, amoxicillin; OX, oxacillin; AMP, ampicillin; IMP, imipenem; KF, cephalothin; CXM, cefuroxime; CTX, cefotaxime; SXT, trimethoprim; LEV, levofloxacin; C, chloramphenicol; E, erythromycin; RA, rifampin; TE, tetracycline; DO, doxycycline; VA, vancomycin; CN, gentamicin; NIT, nitrofurantoin.

All fungal isolates were tested against 7 commercially available antifungal agents (nystatin, fluconazole, itraconazole, amphotericin B, ketoconazole and metronidazole). Eight of the twenty-seven fungal isolates were classified as MDR, and their susceptibility to the antifungal drugs tested is displayed in Suppl. Fig. 2. The mean diameter of nystatin inhibition zones (IZD) ranged from 14.0 ± 0.4 mm (for isolate FI-17) to 20.0 ± 0.05 mm (for isolate FI-21). Suppl. Fig. 2A showed a significant difference ($P < 0.0001$) in susceptibility to nystatin between FI-21 and FI-02, FI-07, FI-10, FI-11, FI-12, and FI-17. Four isolates (FI-07, FI-10, FI-12, and FI-17) showed no sensitivity against fluconazole. As shown in Suppl. Fig. 2B, there was a significant difference in antifungal susceptibility between FI-01 and FI-02 (P 0.001), FI-02 and FI-11 ($P < 0.0001$), and FI-02 and FI-21 ($P < 0.0002$). The mean IZD of itraconazole ranged from 0.0 ± 0.0 mm (for isolate FI-17) to 13.0 ± 0.30 mm (for isolates FI-07 and FI-21), with significance differences (P 0.008 and P 0.0003) in antifungal susceptibility (Suppl. Fig. 2C). For amphotericin B, a significant difference in antifungal susceptibility was observed between FI-01 and FI-02 ($P < 0.0001$), FI-02 and FI-07 ($P < 0.008$), and FI-02 and FI-10 ($P < 0.0001$) as depicted in Suppl. Fig. 2D. Four isolates (FI-07, FI-10, FI-12, and FI-17) showed no sensitivity against fluconazole. A significant difference in antifungal susceptibility was observed between FI-01 and FI-02 ($P < 0.0001$) (Suppl. Fig. 2E). All isolates showed no sensitivity against metronidazole except FI-07 (10.0 ± 0.00 mm) and FI-12 (10.0 ± 0.00 mm), with a significant difference in antifungal susceptibility between FI-02 and FI-07 ($P < 0.0001$) as shown in Suppl. Fig. 2F. Metronidazole outperformed the other antifungal agents tested in mean IZD ($P < 0.0001$). Similarly, there was a significant difference between nystatin and fluconazole (P 0.017), nystatin and itraconazole (P 0.0008), as well as nystatin and amphotericin B (P 0.009). There was no statistically significant difference in mean IZD between ketoconazole and amphotericin B, itraconazole, fluconazole, and nystatin (P 0.291) (Fig. 2).

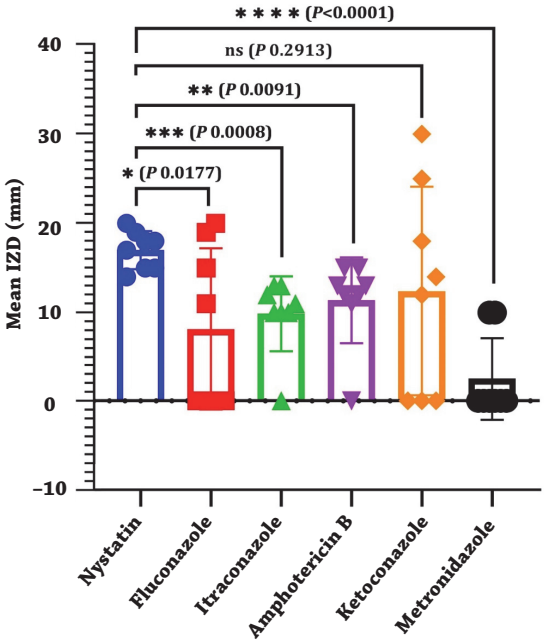

Fig. 2. Comparison of antifungal susceptibility among MDR fungal isolates in terms of mean inhibition zone diameter (IZD)

Antimicrobial activity of acetonic plant extracts against MDR isolates

The antibacterial activity of tested acetonic plant extracts rosemary, henna, and licorice against 21 MDR bacterial isolates was compared to the commercial antibiotic agent gentamicin (positive control) as shown in Table 1. The mean IZDs of rosemary extract ranged from 16.0 ± 0.17 mm (BI-08) to 27.0 ± 0.02 mm (BI-41) (Table 1). The IZDs of henna extract ranged from 14.0 ± 0.11 mm (BI-23) to 25.0 ± 0.05 mm (BI-43) on average (Table 1). Licorice extract IZDs ranged from 16.0 ± 0.0 mm (BI-46) to 31.0 ± 0.25 mm (BI-03) (Table 1). The negative control (solvent) revealed no IZD, whereas the positive control (gentamicin) revealed a range of IZDs (0.0 ± 0.0 to 25.0 ± 0.24 mm) (Table 1). Overall, acetonic rosemary extract outperformed henna and licorice extracts in antibacterial activity (Fig. 3). The mean IZDs of rosemary extract were significantly higher than those of henna and licorice (P 0.037) as well as gentamicin ($P < 0.0001$). As a result, rosemary acetonic extract was chosen to prepare a topical cream for further wound healing experiment. Multiple concentrations of the produced extract were tested against the identified bacterial pathogens

(Fig. 4). The antibacterial activity of acetonic rosemary extract increased with dosage. The extract tested at 400 µg/ml vs. BI-23 had the highest IZD (32.0 ± 0.7 mm). Different acetonic rosemary extract concentrations (100–400 µg/ml) showed a significant difference ($P < 0.0001$) as an antibacterial agent against selected MDR bacterial pathogens (Fig. 4).

Table 1. Antibacterial activities of acetonic plant extracts against different gram negative bacterial isolates

Bacterial isolates Code	# Mean of measured IZ (mm) ± SD obtained using different tested agents			
	Rosemary	Henna	Licorice	CN
BI-01	19 ± 0.02	18 ± 0.02	28 ± 0.22	25 ± 0.24
BI-03	23 ± 0.88	22 ± 0.22	31 ± 0.25	13 ± 0.02
BI-07	25 ± 0.125	19 ± 0.1	27 ± 0.208	20 ± 0.152
BI-08	16 ± 0.17	17 ± 0.34	22 ± 0.1	10 ± 0.03
BI-09	22 ± 0.02	20 ± 0.5	21 ± 0.08	20 ± 0.07
BI-11	21 ± 0.25	19 ± 0.11	22 ± 0.03	0.0 ± 0.00
BI-12	22 ± 0.12	20 ± 0.057	19 ± 0.11	0.0 ± 0.00
BI-17	22 ± 0.88	21 ± 0.22	22 ± 0.02	15 ± 0.12
BI-22	24 ± 0.02	18 ± 0.15	21 ± 0.11	0.0 ± 0.00
BI-23	27 ± 0.66	14 ± 0.11	25 ± 0.07	0.0 ± 0.00
BI-26	23 ± 0.03	18 ± 0.12	23 ± 0.23	0.0 ± 0.00
BI-31	25 ± 0.33	17 ± 0.04	18 ± 0.157	11 ± 0.1
BI-37	26 ± 0.1	19 ± 0.01	19 ± 0.00	12 ± 0.208
BI-38	25 ± 0.33	21 ± 0.22	20 ± 0.2	0.0 ± 0.00
BI-39	23 ± 0.1	20 ± 0.057	19 ± 0.00	14 ± 0.19
BI-40	18 ± 0.00	22 ± 0.01	20 ± 0.208	0.0 ± 0.00
BI-41	27 ± 0.02	20 ± 0.22	21 ± 0.00	18 ± 0.16
BI-43	24 ± 0.33	25 ± 0.05	23 ± 0.02	0.0 ± 0.00
BI-46	20 ± 0.00	23 ± 0.03	16 ± 0.00	11 ± 0.02
BI-47	25 ± 0.152	21 ± 0.11	23 ± 0.02	10 ± 0.03
BI-48	24 ± 0.00	20 ± 0.02	17 ± 0.11	0.0 ± 0.00
Total mean	23.71 ± 3.76	19.71 ± 2.36	21.76 ± 3.65	8.76 ± 8.9

IZ, inhibition zone; SD, standard deviation; CN, gentamicin.

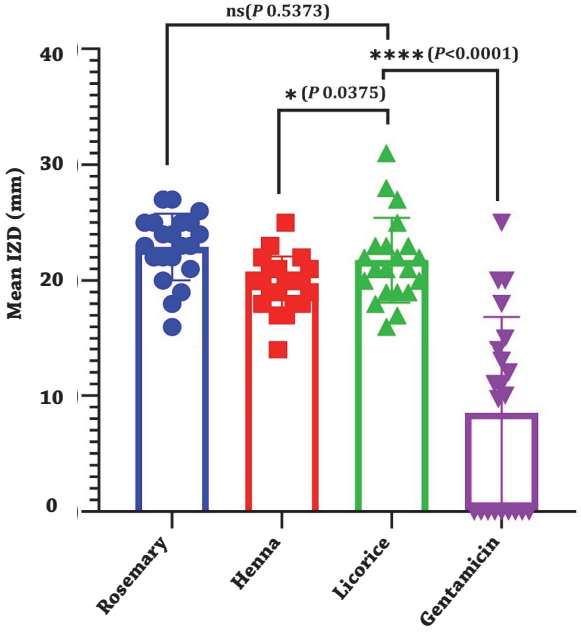

Fig. 3. Comparison of susceptibility of tested bacteria to acetonic plant extracts in terms of mean inhibition zone diameter (IZD)

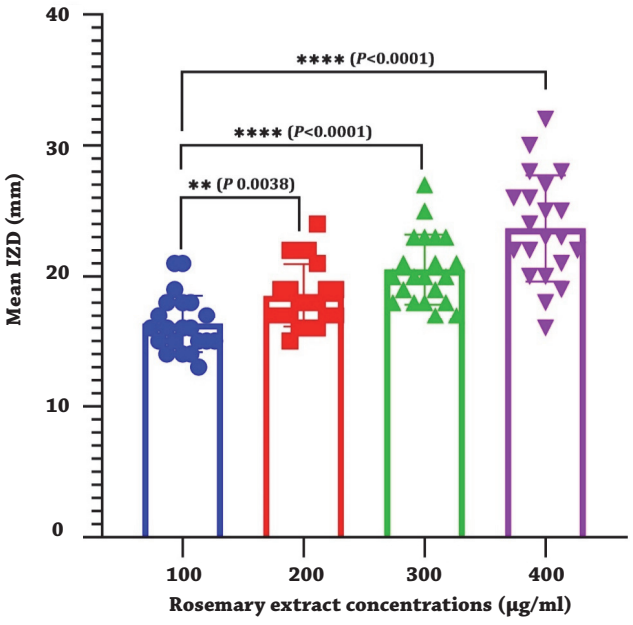

Fig. 4. Minimum inhibitory concentration (MIC) of acetonic rosemary extract against tested MDR bacterial isolates

The antifungal activity of the tested acetonic plant extracts, rosemary, henna, and licorice, against 8 MDR fungal isolates was compared to the commercial antibiotic agent nystatin (positive control) and solvent (negative control) as shown in Suppl. Fig. 3. The mean IZDs of rosemary extract ranged from 14.0 ± 0.5 mm (FI-02) to 25.0 ± 0.3 mm (FI-11) (Suppl. Fig. 3A). The IZDs of henna extract ranged from 17.0 ± 0.1 mm (FI-07) to 23.0 ± 0.0 mm (FI-11) on average (Suppl. Fig. 3B). Licorice extract IZDs ranged from 20.0 ± 0.0 mm (FI-02) to 27.0 ± 0.1 mm (FI-17) (Suppl. Fig. 3C). The negative control (solvent) revealed no IZD, whereas the positive control (nystatin) revealed a range of IZDs (12.0 ± 0.3 to 18.0 ± 0.0 mm) (Suppl. Fig. 3D). Acetonic licorice extract outperformed henna and rosemary extracts in antifungal activity (Fig. 5). The mean IZDs of licorice extract were significantly higher than those of henna and rosemary (P 0.009) as well as nystatin (P < 0.0001). As a result, licorice acetonic extract was chosen to prepare a topical cream for further *in vivo* wound healing and histopathology experiments. Multiple concentrations of the produced extract were tested against the identified fungal pathogens to determine the MIC values of acetonic licorice extract (Fig. 6). The antifungal activity of acetonic licorice extract increased with dosage. The extract tested at 400 µg/ml vs. FI-17 had the highest IZD (31.0 ± 0.03 mm). Different acetonic licorice extract concentrations (100–400 µg/ml) showed a significant difference (P < 0.0001) as an antifungal agent against selected MDR fungal pathogens (Fig. 6).

GC-MS analysis

According to the results of the GC-MS analysis of the rosemary and licorice extracts, the potential antimicrobial activity is attributed to various compounds belonging to a wide range of chemical classes. The higher percentages of Thiocyanic acid, phenylmethyl ester (19.29%), followed by 11.40% in both Bicyclo [2.2.1] .1] heptane-2-one, 1, 7,7-trimethyl-, (1S)- Camphor and Eicosane, 2-methyl-, Heneicosane, 11-(1-ethylpropyl)- in the acetonic extract of rosemary in the acetonic extract of rosemary (Table 2; Suppl. Fig. 4A) are thought to be the cause of this extract's higher antimicrobial activity. As shown in Table 3; Suppl. Fig. 4B, the most abundant compounds in the acetone extract of licorice were 3-O-Methyl-d-glucose (29.07%), followed by Benzenepropanoic acid, 4-hydroxy, methyl ester (12.35%), both of which were associated with high antimicrobial activity.

Molecular identification

Based on the antimicrobial potential of the acetonic plant extracts, rosemary, henna, and licorice, against 21 MDR bacterial isolates and 8 fungal isolates, two isolates, BI-41 and FI-17, were chosen for *in vivo* wound healing and histopathological analysis. As a result, the evolutionary history of the selected isolates BI-41 and FI-17 was deduced using the Neighbor-Joining method (Suppl. Fig. 5). BI-41 stands for molecularly identified species *Pseudomonas aeruginosa* SSM-15, while FI-17 stands for molecularly identified species *Aspergillus niger* SSM-27. The strain SSM-15 showed 90.77% identity to *Pseudomonas aeruginosa* strain YEB L1 (MK263744) (Suppl. Fig. 5A), while SSM-27 showed 95.59% identity to *Aspergillus niger* isolate F5 (MK840965) (Suppl. Fig. 5B).

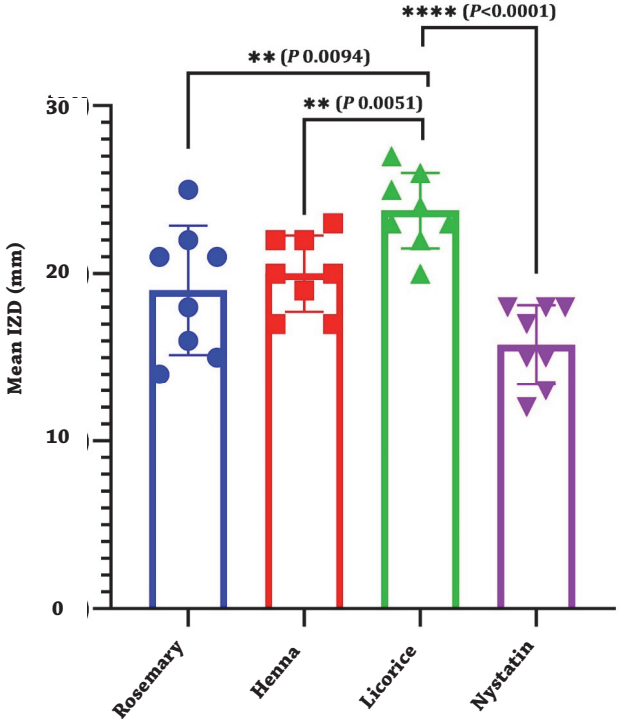

Fig. 5. Comparison of susceptibility of tested MDR fungal isolates to acetonic plant extracts in terms of mean inhibition zone diameter (IZD)

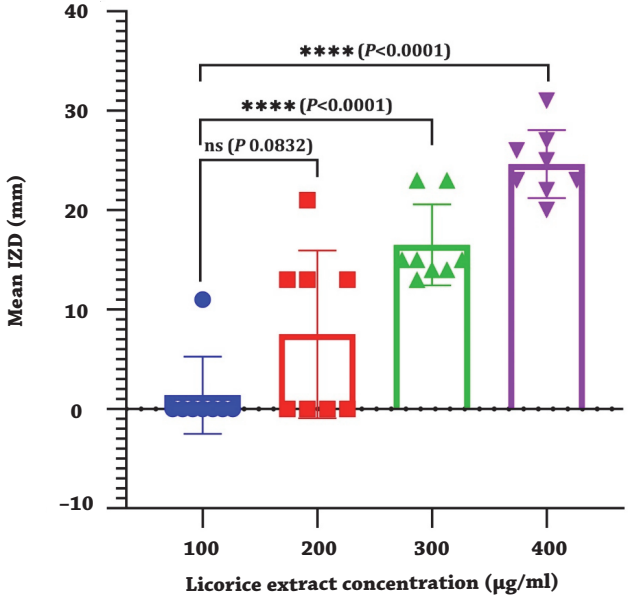

Fig. 6. Minimum inhibitory concentration (MIC) of acetonic licorice extract against tested MDR fungal isolates

Table 2. GC-MS analysis of different compounds of rosemary extract

Peak	RT* (min)	Area%	Compound name
2	16.303	2.857	Eucalyptol
3	20,929	11.408	Bicyclo [2.2.1] heptan-2-one, 1,7,7-trimethyl-, (1S)- Camphor
4	24.84	3.956	Thiocyanic acid, phenyl methyl ester, Hydroxylamine, O-(phenylmethyl)-, Ethanedioic acid
5	27.062	19.29	Thiocyanic acid, phenyl methylester
6	27.827	4.526	Thiocyanic acid, phenyl methyl ester
7	34.505	1.37	Eicosane, 2-methyl-, Tetradecane, 2-methyl-
9	34.780	4.81	Eicosane,Tetracosane, Nonadecane
12	35.795	2.997	Hexadecane, 2,6,11,15-tetramethyl-, Heneicosane
13	36.035	11.076	Eicosane, 2-methyl-, Heneicosane, 11-(1-ethylpropyl)-
14	36.300	1.217	Eicosane, 2-methyl-Hexadecane, 2,6,11,15-tetramethyl-, Nonadecane, 2-methyl-
15	36.371	3.090	Oxirane, hexadecyl-, trans-2-Dodecen-1-ol

* RT, retention time.

Table 3. GC-MS analysis of different compounds of licorice extract

Peak	RT* (min)	Area%	Compound name
1	6.094	0.834	o-Xylene, m-Xylene, p-Xylene
2	6.309	2.896	2-Propanone, 1,3-dihydroxy
3	9.215	0.940	Thymine, phenyl ester
5	10.410	1.740	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-
6	11.366	0.983	Phenylacetaldehyde, Benzene, (ethenyloxy)-
7	11.551	5.010	1H-Pyrazole, 4,5-dihydro-3-methyl-1-propyl-
8	12.256	3.677	Resorcinol
9	12.936	1.502	2-Methoxy-4-vinylphenol
11	15.748	5.749	trans-2-undecenoic acid, Benzene, ethylpentamethyl-
12	16.058	1.045	Ethanone, 1-(2,4-dihydroxyphenyl)-
13	17.138	12.354	Benzenepropanoic acid, 4-hydroxy, methyl ester
15	18.224	29.073	3-O-Methyl-d-glucose
16	18.869	2.194	2,6,10,14,18,22-Tetracosahexaene
18	20.029	2.364	n-Hexadecanoicacid, Palmitic acid, Pentadecanoic acid

* RT, retention time.

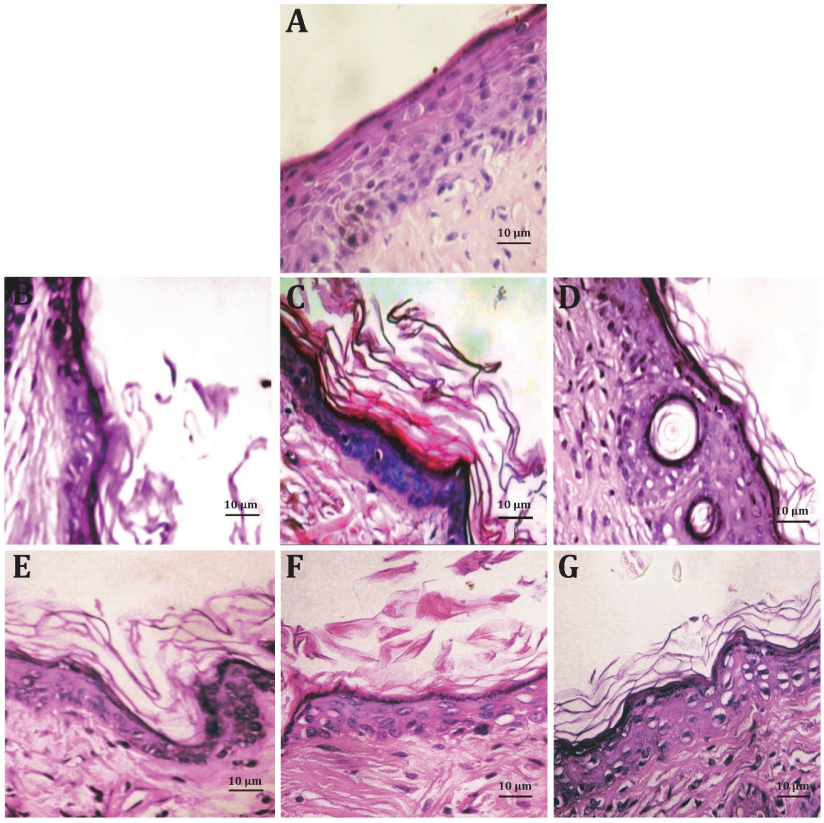

Fig. 7. Histopathological examination of skin mice groups (H&E, ×40). (**A**) Group 1 (G1) mice burned, not infected nor treated; negative control. (**B**) Group 2 (G2) mice burned, infected with *P. aeruginosa* SSM-15, and treated with placebo cream. (**C**) Group 4 (G4) mice burned, infected with *P. aeruginosa* SSM-15, and treated with fucidic acid cream. (**D**) Group 6 (G6) mice burned, infected with *P. aeruginosa* SSM-15, and treated with rosemary extract. (**E**) Group 3 (G3) mice burned, infected with *A. niger* SSM-27, and treated with placebo cream. (**F**) Group 5 (G5) mice burned, infected with *A. niger* SSM-27, and treated with nystatin cream. (**G**) Group 7 (G7) mice burned, infected with *A. niger* SSM-27, and treated with licorice extract.

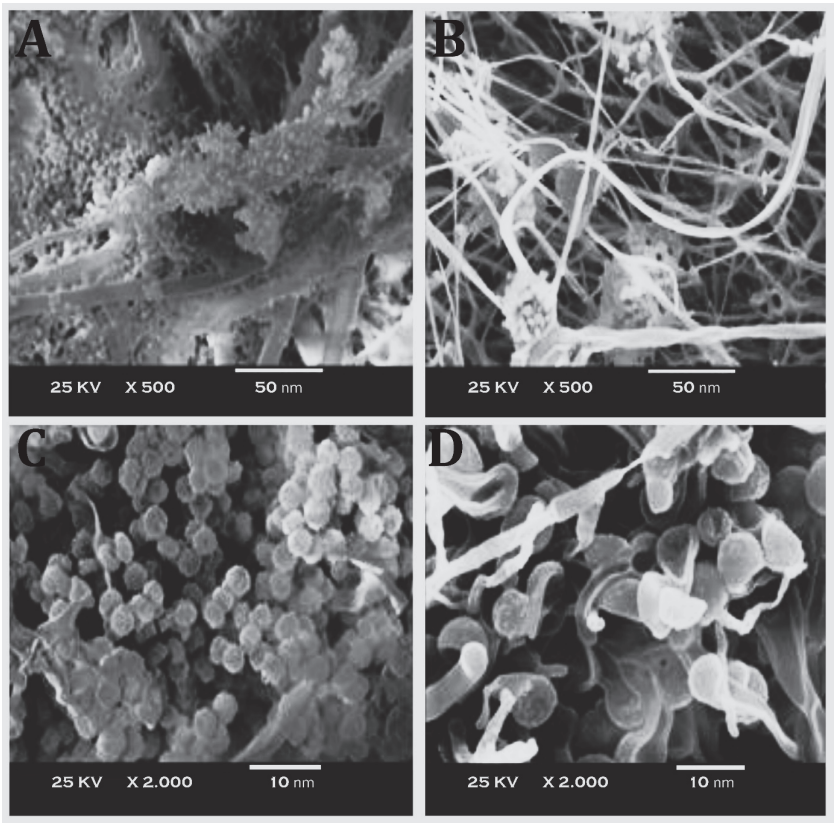

Fig. 8. Scanning electron micrograph (SEM) showing the effect of licorice extract on *A. niger* SSM-27. (**A)** Mycelia of *A. niger* SSM-27 untreated with licorice extract. (**B**) Mycelia of *A. niger* SSM-27 treated with licorice extract. (**C**) Conidia of *A. niger* SSM-27 untreated with licorice extract. (**D**) Conidia of *A. niger* SSM-27 treated with licorice extract.

Efficiency of rosemary and licorice topical formulated creams in wound healing

In mice infected with *P. aeruginosa* SSM-15 or *A. niger* SSM-27, the topical composition of rosemary and licorice acetonic extracts cream demonstrated the highest efficiency with no adverse effects (data not shown). The lesions had vanished after 17 days of topical herbal medicine cream therapy, and there was no sign of irritation. Those who received commercial pharmaceutical cream, on the other hand, developed a small inflammatory lesion.

The effectiveness of a rosemary topical formulated cream in wound healing in mice infected with *P. aeruginosa* SSM-15 is investigated using histopathological analysis (Fig. 7 B–D). The examination of normal skin sections (Group 1(G1); mice were burned but did not become infected or receive any treatment) reveals normal epidermis and dermis layers and normal collagen distribution and secretions throughout the dermal layer (Fig. 7A). Group 2 (G2; placebo cream-treated animals infected with *P. aeruginosa* SSM-15) indicated that the epidermis was damaged, and the dermis was significantly impacted (Fig. 7B). Group 4 (G4; fusidic acid-treated mice; burned; infected with *P. aeruginosa* SSM-15) demonstrated that the epidermis and dermis layers were separated by edema space and extensive infiltration and seemed inflamed with a large number of inflammatory cells (Fig. 7C). Group 6 (G6; acetonic rosemary extract-receiving group; burned mice infected with *P. aeruginosa* SSM-15) treatment resulted in a strong healing effect, preserving the interior skin layers with normal collagen distribution and the emergence of only typical hair follicles, indicating that this formula was beneficial in burn treatments (Fig. 7D).

The effectiveness of licorice topical cream in wound healing in mice infected with *A. niger* SSM-27 is investigated using histopathological analysis (Fig. 7E–G). Group 3 (G3; placebo cream-receiving group; burned mice infected with *A. niger* SSM-27) had round, short, elongated cells, and some hyphal swelling within the stratum corneum, where its keratinized fibers appeared loose and disrupted. The dermis had a chronic inflammatory cellular infiltrate, primarily composed of lymphocytes and plasma cells, as shown in Fig. 7E. The skin section of Group 5 (G5; nystatin-treated mice; burned; infected with *A. niger* SSM-27) had an abnormal epidermis because the stratum corneum's keratinized fibers were still disrupted and some hyphal swelling remained. The dermis showed edema with some inflammatory cellular infiltrate of lymphocytes (Fig. 7F). The burned skin infected with *A. niger* SSM-27 and treated with prepared licorice formula cream (Group 7, G7) showed no hyphal swelling and no significant toxic effects; the skin tissue appeared normal with normal epidermis as keratinized fibers of stratum corneum regularly arranged, appeared condensed without disruption, and the dermis seemed to be normal with minimal inflammatory cellular infiltrate (Fig. 7G). SEM micrographs were used to assess the effect of licorice acetonic extract on the surface morphology of *A. niger* SSM-27 mycelium after three days of treatment at concentrations of 400 mg/ml. As indicated in Fig. 8, hyphae exposed to the selected concentrations of licorice grown on PDA media had degenerative alterations in hyphal morphology compared to the thick, elongated, smooth-surfaced hyphae in the control plates (Fig. 8A, B). In control, the number of conidia was observed (Fig. 8C). However, treatment with licorice extract caused some deformation

within the conidial cell and increased the conidial surface area and constriction of the cell cavity (Fig. 8D).

Discussion

The structural information contained in medicinal plants can be exploited to design novel chemotherapeutic agents (Teka et al., 2022). Anti-infective medication misuse and overuse have increased in recent years, resulting in the emergence of MDR-resistant microbes (Ali et al., 2020b, 2021, 2022). Pathogens developed defence mechanisms that made them more difficult to treat by amassing resistant genes or genetic mutations, prolonging illness, and increasing mortality. In addition, the presence of β-lactamase and ESβL genes have been identified in many MDR *P. aeruginosa* isolates (Sonbol et al., 2015). Traditional drugs have limitations in evaluating safety, quality, and efficacy (Ali et al., 2022). The antibacterial activity of acetonic extracts of rosemary, henna, and licorice, differed between bacterial and fungal isolates. The rosemary and licorice acetonic extracts demonstrated the most effective zone of inhibition against the bacterial and fungal isolates tested. Plant extracts have recently been discovered to have high antibacterial and antifungal activity (Adamczak et al., 2019; Nieto et al., 2018; Veenstra and Johnson, 2021).

According to the GC-MS results, the putative antibacterial action of rosemary and licorice extracts was attributed to several compounds belonging to various chemical classes (Sharma et al., 2011). Higher concentrations of Thiocyanic acid (19.29%) and Bicyclo [2.2.1] heptane-2-one (11.40%) in acetonic rosemary extracts are likely responsible for the extract's improved antibacterial action (Nieto et al., 2018; Veenstra and Johnson, 2021). Because of the extensive layer of peptidoglycan in the cell wall, these chemicals may have synergistic effects, causing damage to both cellular and bacterial membranes (Kumar et al., 2011). The main antibacterial ingredient in licorice acetone extract was 3-O-Methyl-d-glucose (29.07%), followed by Benzene propanoic acid, 4-hydroxy, methyl ester (12.34%). Our findings follow those reported by Bashir et al., 2012. Plant extracts contain several bioactive substances that have antibacterial and antifungal properties. As a result, there is a strong link between the components of plant extracts and their antimicrobial activity (Liu et al., 2017). As shown in Suppl. Fig. 6. the antimicrobial mechanisms of plant extracts, like rosemary and licorice extracts, may also involve disrupting the cell wall and membrane structure and interfering with the energy metabolism and enzyme system. Because of their lipophilic properties, plant extracts can pass through the cell wall and cytoplasmic membrane, exerting cytotoxic effects on living microorganisms. They have been shown to disrupt cell membranes and inhibit the biosynthesis of ergosterol, which is the primary component of the fungal cell membrane (Liu et al., 2017). Cinnamon, for example, has been shown to inhibit bacteria by disrupting cell membranes, changing lipid profiles, and inhibiting ATPase, cell division, and biofilm formation. Cinnamon can also effectively inhibit the growth of gram-positive bacteria *S. aureus* and gram-negative bacteria *E. coli* quickly by altering the cell microstructure (Zhang et al., 2016). Thymol and carvacrol have an enhanced ability to remove lipopolysaccharides and sensitize membranes, allowing them to cause outer cell membrane damage. Because of their ability to release lipopolysaccharides, they have superior antimicrobial properties against some gram-negative bacteria (Omonijo et al., 2018). Cinnamaldehyde can trigger a cascade of apoptotic events in *Aspergillus flavus*, including increased Ca^{2+} and reactive oxygen species (ROS), a decrease in mitochondrial membrane potential, and the release of cytochrome c, and DNA damage. Furthermore, it significantly increased the expression of apoptosis-related genes (Qu et al., 2019).

Infected burn wound mice were treated with topical creams containing acetonic rosemary and licorice extracts. A topical plant formula with high efficacy and no side effects may reduce the need for antibiotic therapy, which is costly and associated with many side effects. When compared to prior treatment and a placebo cream, the results of this study indicated that lesions cleared, and no signs of inflammation were found after about two weeks of topical plant cream therapy. While pharmaceutical cream treatments resulted in small lesions with inflammation, these findings matched those of Shekarchi et al. (2012), who discovered that rosmarinic acid is a phenolic component present in rosemary with significant biological capabilities. Sharma et al. (2011) found that licorice was beneficial against *Candida albicans*. The current study lays the groundwork for future research on herbal plant creams to treat skin ailments.

Conclusions

In conclusion, *in vivo* histopathological examination revealed that acetonic extracts of rosemary and licorice have a high antimicrobial potential against MDR pathogens inhabiting skin burn infections. The ability of herbal treatment to heal infected mice's skin using topical prepared creams demonstrated the potential of herbal therapy as an alternative to commercial antimicrobial agents. More research on human volunteers with skin burns is needed to confirm the efficacy of rosemary and licorice acetonic extracts as novel antimicrobial agents in wound healing, especially given their success in treating burn wounds in experimental animal models compared to commercial synthetic ointment.

Data availability

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Ethics approval

All procedures performed in studies involving human participants followed the ethical standards of Tanta University, as well as the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of interests

All authors declare no competing interests.

Acknowledgements

The authors would like to thank Dr. Shereen F. Gheida, Department of Dermatology and Venereology, Faculty of Medicine, University of Tanta, Egypt, who helped collect patient samples.

Funding

This work was supported by Taif University Researchers Supporting Project number (TURSP-2020/95), Taif University, Taif, Saudi Arabia.

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